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BIOPRODUCTION OF INDOLE 3-ACETIC ACID BY *RHIZOBIUM* STRAINS ISOLATED FROM ROOT NODULES OF *VIGNA TRILOBATA* CULTIVARS

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ABSTRACT

In the present study 25 strains of Rhizobia were isolated from Vigna trilobata cultivars grown in the soils of different districts in Andhra Pradesh. All the 25 strains produced IAA but maximum amount was produced by six strains on Yeast Extract Mannitol (YEM) medium supplemented with L- tryptophan. Among them, three were identified as Agrobacterium sp. and remaining three as one species each of Rhizobium, Sinorhizobium and Ensifer after 16sRNA sequencing. Agrobactrium sp. produced more IAA than rhizobium sp. and others. A. tumifaciens MRR 102 (KC428652) produced maximum amount of IAA (120 µg/ml) while Ensifer sp. MRR125 (KC503885) produced 46.5 µg/ml of IAA. IAA production increased with increase in incubation period from 24h and reached maximum at 72 hours for all the isolates. Similarly, with increase in concentration of L tryptophan, increase in IAA production was observed in all the isolates, with maximum IAA production at 200mg/ml of L tryptophan. IAA production was maximum at pH 7 for all the isolates. These strains were examined for effect of different carbon and nitrogen sources on IAA production. Mannitol proved to be the best carbon source and ammonium sulphate as nitrogen source for highest production of IAA by these isolates.

KEYWORDS: IAA Production Increased, Highest Production of IAA & Yeast Extract Mannitol

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Original Article

INTRODUCTION

Legume rhizobium symbiosis not only helps in fixation of atmospheric nitrogen in nodules and transport to the plant parts but also in synthesis of IAA (Indole acetic acid) in nodules by bacteriods and supply to plant (Ghosh et al., 2008). As a phytoharmone IAA was known to be involved in root initiation, cell division and cell enlargement. IAA producing bacteria are believed to increase root growth and root length, resulting in greater root surface area which enables the plant to access more nutrients from the soil (Biero *et al*., 2007). IAA can protect the plants against external stress conditions (Bianco and Defez, 2009). IAA is a common natural auxin, product of L-tryptophan metabolism in microorganisms and approximately 80 % of rhizosphere bacteria can secrete IAA (Bardish *et al*., 2003). Production of Inode 3- acetic acid (IAA), siderophore and phosphate solubilisation is an important plant growth promoting trait for the rhizobacteria (Arora *et al*., 2001, Deshwal *et al*., 2003). Thus, the rhizosphere bacteria which have the potentiality to exhibit these characteristics were recognised as plant growth promoting rhizobacteria (PGPR).

Further, these PGPR have been reported to increase plant resistance to fungal, bacterial and viral diseases (Maurhofer *et al*., 1986), insects (Zehnder *et al*., 1997) and nematodes (Sikora, 1992).

The genus *Vigna* comprising of nearly 150 species, is one of the major nodulating genera in the family Leguminoseae. *Vigna trilobata* commonly called as Pillipesara, mainly cultivated as short term forage crop in India. A perusal of literature on *Vigna- rhizobium* interactions reveals that the studies on nodulation, isolation, cultural and biochemical studies were carried out mainly on few species of *Vigna* viz., *V. mungo, V. ungiculata* and *V. radiata* only. The studies on cultural and biochemical characterization of the *rhizobium* spp. associated with *V. trilobata* were very merge. On *Vigna trilobata* though nodulation was reported much early in 1930's cultural studies have not been carried out intensively so far. Hence, in the present study it was proposed to carry out detailed study on isolation, characterization and biochemical characteristics of *rhizobium* associated with *V. trilobata*. The present work was also intended to screen the effective strain of *rhizobium* with PGPR characters like IAA production and that can be exploited as bio inoculant, among the six isolates from the *Vigna trilobata* cultivars growing in five geographically distinct areas in Andhra Pradesh.

MATERIALS AND METHODS

Isolation of Rhizobia

Soil samples were collected from agricultural fields under the cultivation of *Vigna trilobata* from all the 25 districts of Andhra Pradesh. Certified seeds of *Vigna trilobata* were purchased from the National Seed Corporation (NSC) Guntur. Plants were raised in earthen pots filled with these district soils and were maintained properly in the Botanical garden of Acharya Nagarjuna University. After 90 days of germination, healthy root nodules from gently uprooted plants, surface sterilized with 0.1% mercuric chloride and 70% alcohol and washed thoroughly by sterile distilled water were used for isolation (Vincent, 1970). Rhizobial strains were isolated from root nodules of *V. trilobata* plants, using selective medium Yeast Extract Mannitol Agar (YEM) with congo red and pure cultures were maintained after sub culturing on the same medium. Pure cultures of all the 25 isolates were authenticated as rhizobia by performing the biochemical tests (Somasegaran and Hoben, 1994) and nodulation ability test on homologous hosts by plant infection tests (Vincent, 1970). Out of the 25, the six strains which produced maximum amount of IAA were further identified up to species level through 16S rDNA sequencing (Macrogen, South Korea) and the sequences were deposited in the gene bank. The strain names with allotted accession numbers used in this study are *Agrobacterium tumifaciens* MRR 102 - KC428652 (isolated from Krishna district soil); *Agrobacterium tumifaciens* MRR 105 - KC428654 (isolated from Khammam district soil); *Rhizobium* sp. MRR 106- KC428655 (isolated from Adilabad district soil); *Agrobacterium tumifaciens* MRR 111 - KC415692 (isolated from Anantapur district soil); *Sinorhizobium* sp.MRR 114 - KC503887 (isolated from Warangal district soil); *Ensifer sp.* MRR125 - KC503885 (isolated from Mahaboobnagar district soil).

IAA Production: The production of IAA was determined by the method Gordon and Weber (1951). For IAA production, all the six strains were inoculated separately in to Erlenmeyer flasks (250ml) containing 100ml of YEB supplemented with L-tryptophan (100mg/ml). The cultures were incubated at 28° C on a rotary shaker at 200 rpm for 72 hrs. After incubation the culture broth was centrifuged at 10,000 x g for 5 min. and used for IAA extraction (Sinha and Basu, 1981). To the 10 ml of the supernatant, 2 ml of 2%Salkowsky's reagent (0.5 M $FeCl_3$ in 35% perchloric acid) was added and incubated for 30 min. under darkness. The absorbance of the colour developed was measured at 530 nm in a spectrophotometer. The amount of IAA produced was calculated by using the standard graph of authentic IAA (Hi-media).

Effect of L-Tryptophan Concentrations

The effect of L-tryptophane concentrations on IAA production was studied using YEM medium supplemented with L-tryptophane concentrations of 50, 100, 150, 200, 250 and 300 mg/ml. All the six isolates were tested for IAA

production at different concentrations separately after inoculation and incubation for 72 hours at room temp.

Effect of pH

All the six isolates were tested for IAA production at different pH concentrations (5,6,7,8, and 9) using YEM medium supplemented with (100mg/ml) L- tryptophan.

Effect of Incubation Period

Effect of incubation period on IAA production was studied by inoculating *Rhizobium* isolates separately into L-tryptophan (100mg/ml) supplemented YEM medium and incubated at different periods with 24 hrs intervals up to 144 hrs at 30± 2^{o}C.

EFFECT Of CARBON AND NITROGEN SOURCES

To study the effect of carbon sources on IAA production, 10 different carbon sources viz., Mannitol, Glucose, Lactose, Sucrose, Raffinose, Maltose, Arabinose, Galactose, Fructose, Xylose were added separately to L-tryptophane (100mg/ml) containing YEM medium in equal concentration of Mannitol (1%) of the original YEM medium composition. These flasks were inoculated with the six isolates separately and incubated at room temperature on a rotary shaker at 200 rpm for 72 hrs. After incubation the amount of IAA produced was estimated by standard method (Gordon and Weber, 1951).

To study the effect of nitrogen sources on IAA production, six different nitrogen sources (Ammonium Sulphate, Glysine, Ammonium Nitrate, Sodium Nitrate, L-asperagine, Tyrosine) were inoculated into YEB medium containing L-tryptophan (100mg/ml) and incubated at room temperature on a rotary shaker at 200 rpm for 72 hours. The amount of IAA produced was estimated calorimetrically by standard method (Gordon and Weber, 1951).

Three replicates were maintained for each treatment. Statistical analysis of the data was performed using SPSS software (version 20). ANOVA and Duncan's multiple test were carried out as per the data and results were considered to be significant at $P<0.05$.

Structural characterization of IAA was done for one of the isolates in this study. *Agrobacterium tumifaciens* MRR 102 was inoculated into 200 ml of YEM medium and incubated at room temperature for 3 days on a rotary shaker. After incubation, bacterial cells were separated from the supernatant by centrifugation at 10,000 rpm for 30 min. The supernatant was acidified to pH 2.5-3.0 with 1N HCl and extracted twice with ethyl acetate (Sinha and Basu, 1981).The crude extract was partially purified by silica gel column chromatography and the indole containing fraction was further purified via preparative high-performance liquid chromatography. The pure compound obtained was structurally identified by nuclear magnetic resonance spectroscopy (^{1}H NMR), ^{13}C NMR and Electron Ionization Mass Spectra (EI-MS) (Fig 1,2,3).

RESULTS AND DISCUSSIONS

Spectral Data of IAA: In EIMS, the compound showed molecular ions at *m/z* = 176 inferring a molecular weight of $C_6H_{15}O_2$ $[M+1]^+$. The proton NMR of the compound displayed protons at 3.70δ (Sharp, S, 2H), 7.0δ (t-aromatic-C-H), 7.10δ (t-aromatic-C-H), 7015δ (S-C-H-broad), 7.32δ (d-aromatic-C-H) and 7.51δ (d-aromatic-C-H). ^{13}C NMR depicted peaks at 30.59(s, C-10), 107.48 (s, C-3), 110.84 (s, C-7), 118.05 (s, C-6), 118.42 (s, C-2), 121.04 (s, C-4), 123.22 (s, C-5), 127.27 (s, C-8), 136.61 (s. C-9), 175.06 (s, C-11). Based on the above spectral and mass data, the pure compound obtained

from the culture filtrate of the strain was structurally confirmed as Indole-3-acetic acid.

IAA Production: In the present study, highest IAA production of 120µg/ml was observed in *A. tumefaciens* MRR102 followed by70 µg/ml in *Sinorhizobium* sp. MRR 114. The remaining strains viz., *Rhizobium* sp.MRR 106, *Ensifer* sp. MRR 125 and *A. tumefaciens* MRR 111 produce about 50 µg/ml of IAA. *Ensifer* sp. from soybean was reported to produce a maximum of 30.90 µg/ml of IAA in presence of L tryptophan (0.01%) (Kaur et al, 2014). All the six isolates showed progressive increase in IAA production with increase in incubation period (Table 1) until they reach the stationary phase at 72 hours. After reaching the peak at 72 hrs, a decline in IAA production was observed. This decrease in IAA level was due to the release of IAA degrading enzymes such as IAA oxidase and peroxidase in *Rhizobium* sps. from *Cajanus cajan* (Datta and Basu,1998).

L-tryptophan positively induced the synthesis of IAA in all the isolates studied. The IAA production was increased with increase in L- tryptophan concentration up to 200mg/ml in all the isolates and decreased afterwards (Table 2). This decrease in IAA production after the peak appearance was reported by many workers (Ahmed et al, 2008). Ghosh and Basu (1997) reported an increase in IAA production up to 3% L-tryptophan. Though L- tryptophan was preferred by these isolates, the concentration of L tryptophan doesn't directly influence the amount of IAA produced was previously reported by Bhattacharya (2006) that a maximum of 250 µg/ml of IAA was produced by *Rhizobium* sp. at 2% L-tryptophan while *Rhizobium* sp. from *Derris scandens* produced 135.2 µg /ml of IAA in presence of 4mg/ml of L tryptophan (De and Basu 1995). *Rhizobium* sp. from *Melilotus alba* (Datta and Basu, 1998) produced 190 µg/ml of IAA with 3mg/ ml of L-tryptophan. However, the amount of IAA produced and was much lesser than the amount of L-tryptophan applied in all these studies including the present study was due to its utilization in the synthesis of other indole compounds and also in protein synthesis (Bhowmick and Basu, 1986). Previous literature shows, much variation among the Rhizobial isolates regarding IAA production. Mandal *et al* (2007) reported 40 mg/L of IAA by *Rhizobium* sp. from *V. mungo*. Patel *et al* (2012) reported only 35.67 µg/ml of IAA at by *Bradyrhizobium yuanmingense* from *Abrus precatorius.* In the present study, variation was observed not only between the species but also within the same species. Maximum IAA production of 142µg/ml was recorded in *A. tumefaciens* MRR102 while only 72µg/ml in *A. tumefaciens* MRR111.

All the six Rhizobial isolates produced maximum IAA at pH 7 (Table 3). Similarly majority of the previous workers reported the similar pH range for the maximum IAA production. Mandal *et al* (2007) reported pH7.2 as optimum for *Rhizobium* from *Vigna mungo* and Bhattachraya (2006), Madhuri, (2011) and Apine, (2011) reported the pH in the range 7.0 – 7.2 for *Rhizobium* sp.

In the present study, *Agrobacterium* sp. (*A. tumefacience* MRR102) produced more IAA than *Rhizobium* sp . (MRR106). However, Shokri and Emtiazi (2010) reported that *Rhizobium* sp. produced more amount of IAA than *Agrobacterium* sp.

Effect of Carbon and Nitrogen Sources: Mannitol, among the carbon sources tested, was proved to be the best for maximum production of IAA by all the six *rhizobium* isolates (Table 4). Among the isolates, *A. tumefaciens* MRR102 produced maximum of 120 µg/ml of IAA in YEM containing mannitol with 100mg/ml of L–tryptophane after 72 hours of incubation. Mannitol supporting maximum IAA production was also reported by many workers including De and Basu (1995) from *Rhizobium* sp. of *Derris scandens* and by *Rhizobium* sp. from *Phaseolus mungo* (Ghosh et al., 2008), *Rhizobium* sp. from *Dalbergia lanceolaria* (Ghosh and Basu, 2002) by *Bradyrhizobium yuanmingense* from *Abrus precatorius* (Patel et al., 2012) and by *Rhizobium undicola* from *Neptunia oleracea* (Ghosh et al, 2015). After mannitol,

disaccharides sucrose was preferred by *A. tumefaciens* MRR102 and lactose by *A. tumefaciens* MRR105, *A. tumefaciens* MRR111and *Sinorhizobium* sp. MRR125. Among the monosaccharides used, glucose was preferred next to mannitol by *Rhizobium* sp MRR 106. Variation in carbon source preference by rhizobial isolates for IAA production was also reported previously. Sucrose was preferred by Rhizobial isolates (Ghosh and Basu, 1997), Glucose by *Rhizobium* sp. from *V. mungo* (Mandal et al, 2007), *Rhizobium* sp. from *Cajanua cajan* (Datta and Basu, 2000); Lactose by rhizobium sp. from *Melilotus alba* (Datta and Basu, 1998) (Madhuri, 2011). Utilization of wide range of carbon sources is a good trait for the rhizobial species for better survival in the soil environment. All the six strains in the present study efficiently utilized all the 10 different carbon sources tested and produced the IAA more than that in control condition without any carbon source. The interaction between rhizobial strains and carbon sources was statistically significant ($p<0.05$)

Majority of the strains preferred ammonium nitrate as nitrogen source for maximum IAA production (Table 5). A tenfold increase in IAA production over the control was observed in all these strains. Sodium nitrate was preferred by *A. tumefaciens* MRR102 and *Rhizobium* sp MRR106. Ammonium nitrate supported the highest IAA production in the remaining isolates. This result coincides with the previous reports. Glutamine, glutamate, or NH_{4+} was generally preferred as nitrogen source by some strains of *Rhizobium* spp. (Chakrabarthy *et al*, 1981). Nitrate preference by *rhizobium* sp for IAA production was previously reported by many workers. Sodium nitrate was effective in *rhizobium* sp. (Ghose and Basu, 1997); Potassium nitrate was preferred by *rhizobium* sp. from *Derris scandens* (De and Basu, 1995), *Dolichos biflorus* (Datta and Basu, 1997),*Cajanus cajan* (Shinde and Patel, 2011) and *V. mugo* (Mandal et al, 2007). Glycene, L-asparagine and tyrosine are least preferred by the strains of *V. trilobata* for IAA production in this study, while L-asparagine supported the maximum IAA production in rhizobia isolated from *Alysicarpous vaginalis* (Bhattacharya and Pati, 2000) and in *Rhizobium* sp. from *Phaseolus mungo* (Ghosh et al., 2008).

CONCLUSIONS

From the present study it is clear that rhizobial strains from *V. trilobata* preferred mannitol as carbon source and nitrates as nitrogen source for the production of IAA with optimum concentration of 200mg/ml L-tryptophane at pH 7.0 after 72 hours of incubation. That the IAA production by rhizobacteria varies among different species and strains, and also influenced by cultural conditions, growth stage and substrate availability, was clearly evidenced from the present study.

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APPENDICES

Table1: Effect of Incubation Period on IAA Production by *Rhizobium* Strains of *Vigna trilobata*

Incubation periods (Hours)	*Agrobacterium tumifaciens* MRR 102	*Agrobacterium tumifaciens* MRR 105	*Rhizobium* sp. MRR 106	*Agrobacterium tumifaciens* MRR 111	*Sinorhizobium* MRR 114	*Ensifer* sp. MRR 125
24 hrs	22	15	19	11	13	31
48 hrs	25	25	28	20	18	36

Table 1 Contd.						
72 hrs	120	52	55	46.5	70	46.5
96 hrs	63	25	24	18	23	25
120 hrs	07	05	06	06	06	04

* The F-value for incubation period and interaction are significant with $p<0.05$.

Table 2: Effect of L-Tryptophan Concentrations on IAA Production (µg/Ml) by *Rhizobium* Strains of *Vigna trilobata*

L-Tryptophan Concentration (Mg/Ml)	*Agrobacterium Tumifaciens* MRR 102	*Agrobacterium Tumifaciens* MRR 105	*Rhizobium* Sp. MRR 106	*Agrobacterium Tumifaciens* MRR 111	*Sinorhizobium* MRR 114	*Ensifer* Sp. MRR125
50	56	27	38	37	30	22
100	120	52	55	46.5	70	46.5
150	135	80	73	52	107	73
200	142	92	85	72	142	86
250	42	24	25	25	29	20
300	15	06	05	06	06	06

*****The F-value for L Tryptophan, *Rhizibium* strains of *Vigna trilobata* and interaction are all significant with $p<0.05$.

Table 3: Effect of Ph on IAA Production by *Rhizobium* Strains of *Vigna Trilobata* at (100mg/Ml) L tryptophane)

pH	*Agrobacterium tumifaciens* MRR 102	*Agrobacterium tumifaciens* MRR 105	*Rhizobium* sp. MRR 106	*Agrobacterium tumifaciens* MRR 111	*Sinorhizobium* MRR 114	*Ensifer* sp. MRR125
pH -5	**31**	**34**	**26**	**30**	**20**	**30**
pH -6	**38**	**51**	**44**	**44**	**51**	**44.5**
pH -7	**120**	**52**	**55**	**46.5**	**70**	**46.5**
pH -8	**45**	**44**	**40**	**46**	**46.5**	**39**
pH -9	**20**	**20**	**20**	**33**	**30**	**20**

*The F-value for pH and interaction are significant with p<**0.05.**

Table 4: Effect of Carbon Sources on IAA Production by *Rhizobium* Strains of *Vigna trilobata*

Carbon Sources (1.0%)	*Agrobacterium tumifaciens* MRR 102	*Agrobacterium tumifaciens* MRR 105	*Rhizobium* sp. MRR 106	*Agrobacterium tumifaciens* MRR 111	*Sinorhizobium* MRR 114	*Ensifer* sp. MRR125
Control	**16**	**15**	**12**	**06**	**11**	**6.5**
Mannitol	**120**	**52**	**55**	**46.5**	**70**	**46.5**
Glucose	**54**	**24**	**46**	**27**	**20**	**23**
Lactose	**32**	**36**	**25**	**31**	**32**	**37**
Sucrose	**90**	**23**	**25**	**16**	**23**	**23**
Raffinose	**25**	**13**	**26**	**31**	**35**	**37**
Maltose	**28**	**36**	**22**	**14**	**46**	**37**
Arabinose	**44**	**21**	**26**	**02**	**12.5**	**28**
Galactose	**32.5**	**29**	**15**	**18.5**	**29**	**14**
Fructose	**17**	**27**	**31**	**20**	**16**	**07**
Xylose	**27**	**24**	**29**	**05**	**13**	**14**

* The F-value for carbon sources, Rhizibium strains of Vigna trilobata and interaction are all significant with $p<0.05$.

Table 5: Effect of Nitrogen Sources on IAA Production by *Rhizobium* Strains from *Vigna trilobata*

Nitrogen Sources (0.1%)	*Agrobacterium tumifaciens* MRR 102	*Agrobacterium tumifaciens* MRR 105	*Rhizobium* sp. MRR 106	*Agrobacterium tumifaciens* MRR 111	*Sinorhizobium* MRR 114	*Ensifer* sp. MRR125
Control	**06**	**05**	**05**	**2.5**	**2.5**	**5**
Ammonium Sulphate	**48**	**23**	**29**	**30**	**30**	**35**
Glysine	**7.5**	**05**	**10**	**10**	**04**	**06**
Ammonium Nitrate	**50**	**46**	**29**	**37**	**43**	**43**
Sodium Nitrate	**62**	**40**	**37**	**32.5**	**35**	**35**
L-Asperagine	**15**	**12.5**	**15**	**08**	**10**	**10**
Tyrosine	**15**	**12**	**12.5**	**20**	**15**	**15**

*The F-value for Nitrogen sources, Rhizibium strains and interactions all are significant with $p<0.05$.

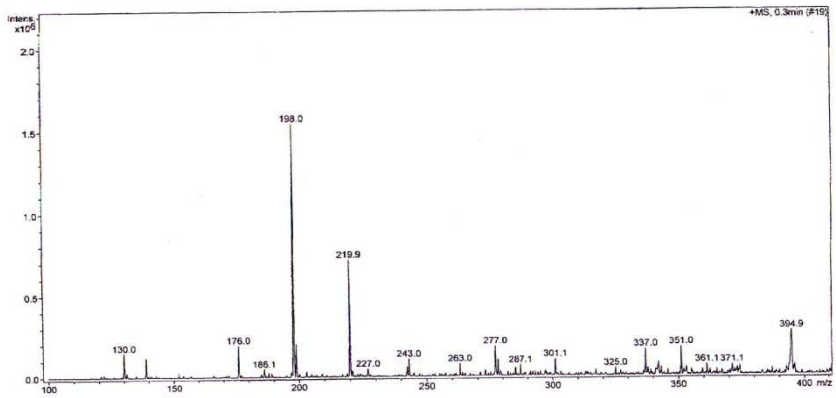

Figure 1: Electron Ionization Mass Spectrum of Indole-3-Acetic Acid Produced by *A. tumifaciens* MRR 102

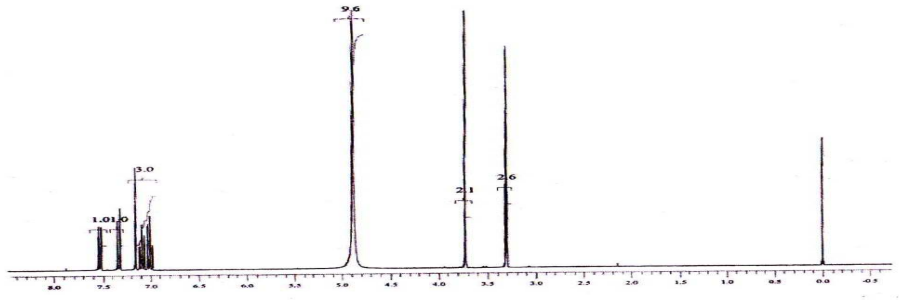

Figure 2: ^{1}H NMR of Indole-3-Acetic Acid Produced by *A. tumifaciens* MRR 102

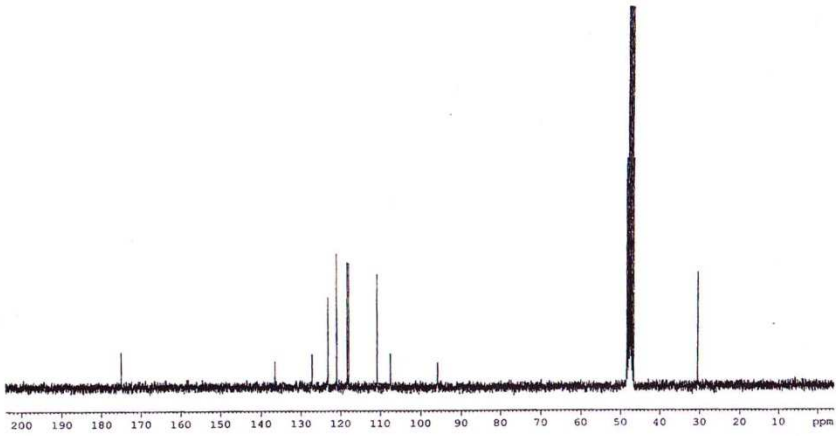

Figure 3: ^{13}C NMR of Indole-3-acetic acid produced by *A. tumifaciens* MRR 102

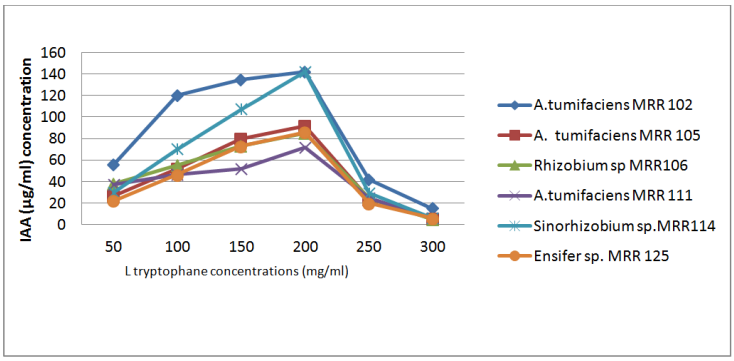

Figure 4: Effect of L-Tryptophane Concentraion on IAA Production

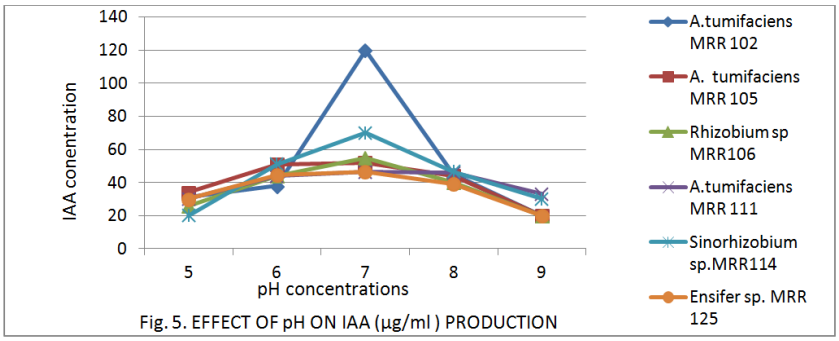

Figure 5: Effect of Carbon Sources on IAA Production

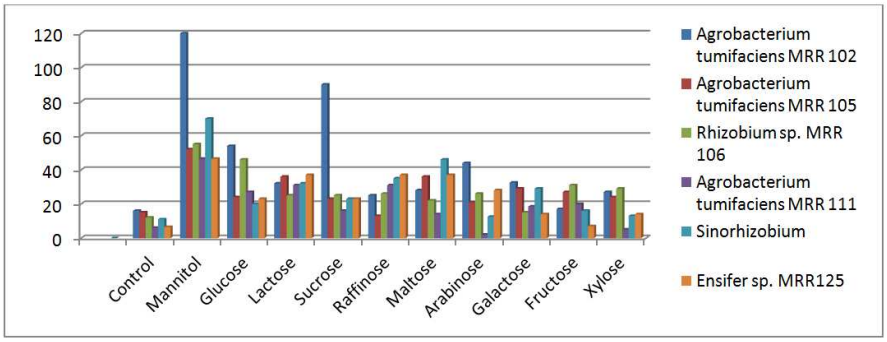

Figure 6

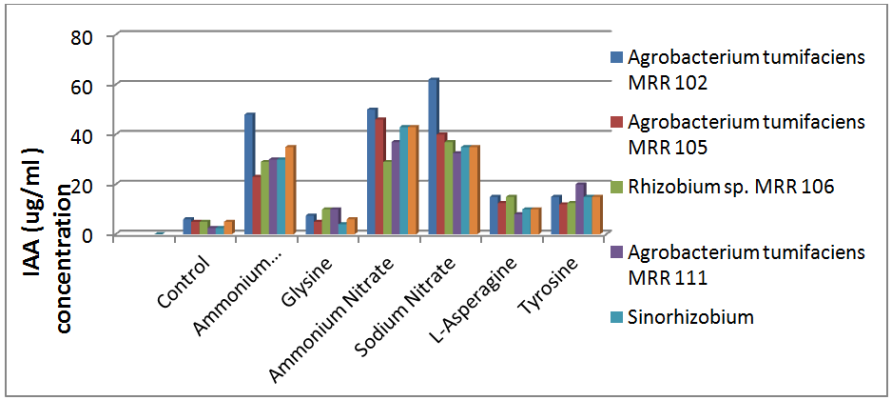

Figure 7: Effect of Nitrogen Sources on IAA Production